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13. ABSTRACT ***(Maximum 200 Words)***

This application proposed to test two hypotheses. The first hypothesis to be tested in this was whether c-*myb* and A-*myb* genes regulate ductal cell proliferation and whether abnormalities in the expression of these genes results in breast cancer. Our studies employed both in vitro and in vivo systems. During the first phase of the granting period, we developed embryonic stem cells to generate mice that either have breast-specific deletion of c-myb gene or breast-specific truncation of c-myb gene leading to its activation. In addition, we developed in vitro systems to test the oncogenic behavior of c-myb and A-myb genes. These results show that over-expression of c-myb or A-myb leads to the transformation of breast epithelial cells validating our hypothesis that these two genes constitute excellent targets for drug development. Analysis of human breast tumor cell lines showed that c-myb is expressed in a majority of ER+ human breast carcinomas, while A-myb expression is seen predominantly in ER- cell lines. Our results also show that blockage of the biochemical function of c-myb results in a complete block to ER+ breast tumor cell proliferation. The second hypothesis we tested was whether STAT family of transcription factors play a role in the development of ER-negative breast carcinomas. For this, we have examined the activation status of two STATs, STAT-3 and STAT-5. Our results show that several of the ER-negative breast carcinomas express constitutively activated Src kinases, which mediate the phosphorylation of STAT-3, which in turn could mediate their proliferative function and oncogenicity. The constitutively activated status of STAT-3, therefore serves as a good diagnostic marker for the detection of ER-negative breast carcinoma development.

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A. INTRODUCTION

The *myb* gene family currently consists of three members, named A,B and c-*myb*, all of which encode nuclear proteins that bind DNA in a sequence-specific manner and function as regulators of transcription (1). Of these, c-*myb* has been studied extensively in cell culture systems of differentiation and transformation and during mouse development. These studies have provided evidence for the crucial function of the c-*myb* gene in the development, proliferation and oncogenesis of the hematopoietic system (1). A series of recent experiments have implicated a role for this gene family in breast development and breast cancer (2). The first evidence that implicated a role for c-*myb* in breast tumors came from the observation that this gene is highly expressed in all estrogen receptor positive (ER+) breast tumors (2). Our *in situ* hybridization studies also show that this gene is highly expressed in proliferating epithelial cells of the ductal epithelium, in virgin as well as pregnant mice, suggesting that this gene, like in the hematopoietic cell system, might play a critical role in the proliferation of these cells (3). Our *in situ* hybridization studies also show that A-*myb* is not expressed, or is expressed at very low levels, in ductal cells derived from virgin mice. These levels, however, increase dramatically during the cell proliferation that accompanies pregnancy, resulting in ductal branching and alveolar development. Since this phase of ductal branching is mainly induced by the combined action of estrogen and progesterone, these results suggest that A-*myb* might play an important role in this phase of ductal cell proliferation and morphogenesis into alveolar structures. This view was further supported by our studies where we developed A-*myb* null mutant mice, which were unable to produce milk and showed defects in mammary tissue proliferation following pregnancy (4). The loss of A-*myb* expression seems to result in a loss or diminution of progesterone-induced proliferative events associated with the pregnancy-induced morphogenesis of breast tissue (4).

Based on these studies, we hypothesized that c-*myb* and A-*myb* genes play a pivotal role in the proliferation of breast epithelial cells in the adult mice. It is our hypothesis that proliferative events associated with ductal cell proliferation of virgin mice is mediated by c-*myb* and that proliferative events associated with ductal branching and alveolar development that occur following pregnancy are mediated by the combined action of c-*myb* and A-*myb*. It is also our contention that proper down regulation of A-*myb* and c-*myb* are essential for apoptotic events associated with involution. Events that lead to deregulation of expression of c-*myb* and A-*myb* might represent a point at which the onset of neoplasia, which, in combination with other mutations and deletions in oncogenes and growth suppressor genes, results in a metastatic disease. A-*myb* null mutant mice have been extremely useful to our understanding of the role of A-*myb* in mammary gland development (4). Unfortunately, however, c-*myb* null mutant mice have not been very useful to study the role of this gene in mammary gland development since these mice die in utero due to failure of fetal hepatic hematopoiesis (5). To address the role of c-*myb* in mammary development, we proposed to develop c-*myb* mutant mice where the expression of this gene is interrupted specifically in the mammary gland using the Cre-*lox* system.

Task 1: Months 1-12: During this period we proposed to construct targeting vectors that can be used for the deletion of exons 6 and 9 of mouse c-*myb* genes. These vectors were to be transfected into embryonic stem cells and G418 resistant clones were to be screened for homologous recombination. Following the selection of the clones, the cell lines were to be subjected to transient Cre expression and selection for ganciclovir-resistant clones.

B. PROGRESS REPORT

To determine the role of c-myb and A-myb in breast epithelial cell proliferation, we first carried out in situ hybridization studies (**Fig. 1**). Our *in situ* hybridization studies showed that c-myb gene is highly expressed in proliferating epithelial cells of the ductal epithelium, in virgin as well as pregnant mice, suggesting that this gene, like in the hematopoietic cell system, might play a critical role in the proliferation of these cells. Our *in situ* hybridization studies also show that A-*myb* is not expressed, or is expressed at very low levels, in ductal cells derived from virgin mice. These levels, however, increase dramatically during the cell proliferation that accompanies pregnancy, resulting in ductal branching and alveolar development. Since this phase of ductal branching is mainly induced by the combined action of estrogen and progesterone, these results suggest that A-*myb* might play an important role in this phase of ductal cell proliferation and morphogenesis into alveolar structures. This view was further supported by our studies where we developed A-*myb* null mutant mice, which were unable to produce milk and showed defects in mammary tissue proliferation following pregnancy. The loss of A-*myb* expression seems to result in a loss or diminution of progesterone-induced proliferative events associated with the pregnancy-induced morphogenesis of breast tissue.

To determine the role of these two oncogenes, we examined the effect of their constitutive expression during proliferation and differentiation of mouse HC11 mammary epithelial cells. We find that, as with other cell types, c-*myb* mRNA expression is tightly correlated with proliferation and is associated predominantly with G1 phase, with marked downregulation when cells are induced to differentiate **(Fig. 2).** The A-*myb* mRNA, by contrast, is expressed most abundantly during the late S/G2 phase of the cell cycle and is also downregulated, although to a lesser extent than c-*myb*, during differentiation. Unlike c-*myb*, the A-*myb* gene remains responsive to stimulation with a combination of serum, hydrocortisone and prolactin, even in non-proliferative confluent cultures. These results indicate a likely role for c-*myb* and A-*myb* in promoting cell proliferation in response to different hormonal and environmental stimuli. In addition, deregulated expression of both c-*myb* and A-*myb* led to anchorage independent growth, suggesting that both these oncogenes are capable of transforming mammary epithelial cells **(Fig. 3).** Although a normal pattern of Stat5 activation was observed (**Fig. 4**), cells with constitutive expression of c-*myb* and A-*myb* were blocked in the lactogenic induction of ß-casein gene transcription **(Fig. 5).**

To generate breast-specific *c-myb* null genotype in mice, we have isolated a Bam H1 genomic clone of *c-myb* encoding exons 2 through 8, which was used to produce a gene targeting vector (**Fig. 6**). A 0.9 kb fragment containing exon 6 was generated by PCRamplification and cloned into the BamHI site of the *pflox* vector. The flanking 6.0 kbp fragment on the 5' end containing exons 2-5 was generated by a combination of PCR and restriction enzyme digestion and cloned into the SalI site. Similarly, the flanking 2.5 kb fragment on the 3' end containing exons 7-8 was generated by a combination of PCR and restriction enzyme digestion and cloned into the XhoI site. Following the construction of the targeting vector, the entire clone was sequenced to ascertain that PCR technique did not produce any mutations or deletions.

The targeting construct, linearized by NotI digestion, was introduced into ES cells via electroporation. ES cells were plated on gelatin coated culture plates and selected for 10 days with medium containing 150 µg/ml of active G418. This procedure yielded approximately 600 clones. Genomic DNA from these ES clones was digested with Stu1 and homologous recombinants identified by Southern blotting using *c-myb* specific probe downstream of exon 9. Homologous recombinants **(Fig. 7)** revealed two bands: 11.5 kb (wt) and 12.6 kb (recombinant). In order to verify that the recombinants retained all the three *loxP* sites, DNA digested with EcoRI was analyzed by Southern blotting using *loxP* specific probe. All the positive recombinants revealed three bands of sizes 0.3 kb, 1.7 kb and 2.1 kb indicating the presence of 5' *loxP*, internal *loxP* and 3' *loxP* sites respectively.

To produce ES cell subclones that contained the Type 1 (systemic) and Type II (conditional) *c-myb* mutations, ES clones were subjected to transient Cre expression by electroporation of supercoiled pPGK-Cre-bpA and subsequent selection in the presence of ganciclovir. Subclones resistant to ganciclovir were isolated and analyzed by genomic Southern blotting. Using the genomic probe on DNA digested with BamHI, Type II deletions were verified by identifying a band of 6.0 kbp. Type I deletions would reveal a band of 11.5 kb, close to the wild-type band. To further confirm the deletions, DNA digested with BamHI was probed with *loxP* to identify two bands of sizes 6.0 kb and 2.5 kb (Type II) or a single band of size 11.5 kb (Type I).

We have microinjected the *c-myb*^{F} (Type II) ES cell clones into C57BL/6 blastocyst-stage embryos. Todate several chimeric mice have been produced and are currently being used to generate heterozygous mice for the *c-myb*^{F} allele by crossing chimeric mice withc57/B6 mates. Germline transmission is being assessed by digesting tail DNA with BamHI and Southern blotting using the genomic probe to reveal two bands: 11.5 kb (wt) and 6.0 kb (recombinant). We plan to investigate deletion of *c-myb* gene in breast tissue using transgenic mice expressing Cre recombinase specifically in breast tissue. Dr. Lothar Hennighausen of NIH, under a collaborative agreement has agreed to provide us with two sets of transgenic mice, with the Cre recombinase under the control of Whey Acidic Protein (WAP) promoter and MMTV promoter. In these mice, the Cre recombinase is not expressed in any tissue other than the breast. Even in the breast tissue, the recombinase is not expressed in mammary tissue of

virgin mice and the first expression of the recombinase was seen at day 14 of pregnancy and increased during lactation. Interestingly, the recombinase activity could be detected 30 days after weaning, suggesting that the transgene has been active in putative stem cells. These mice would be ideal to determine whether c-*myb* presence is necessary for breast epithelial cell proliferation during pregnancy and alveolar development and if the expression of this gene is necessary for ductal morphogenesis following involution.

To validate the results obtained from the mouse models in the human system, we have conducted parallel studies with human breast tumor cell lines. Analysis of several human breast tumor cell lines showed that the c-*myb* is expressed in a vast majority of ER+ human breast carcinoma cell lines, while A-*myb* expression is seen predominantly in ER- cell lines. To establish the role of c-*myb* in ER+ cell proliferation, we have constructed dominant negative mutants of c-Myb, which effectively block c-*myb*-mediated transactivation of target genes. Expression of these dominant negative mutants in ER+ breast tumor cell lines resulted in complete block to their proliferative potential. In addition, these tumor cell lines lost their tumorigenic activity as evidenced by their inability to grow in soft agar. These results firmly establish that c-*myb* gene plays an essential role in the proliferation of human breast carcinomas. We are currently in the process of assessing the role of A-*myb* in the proliferation of ER- breast carcinomas.

Tasks 2-4 were not performed as a similar application was funded by NIH and I made a request to the US Army to allow me to use the remaining funds to study the role of STAT-3 in ER-negative breast tumors. This request was approved and so I switched the focus of the work to study the role of STAT-3 in the development of ER-negative breast tumors.

Introduction for STAT-3 work:

STATs (Signal Transducers and Activators of Transcription) are a group of highly related transcription factors, which were originally described by Darnell and his co-workers as transcription factors induced by interferons (6). Subsequent studies by a number of groups showed that STATs play a critical role in signal transduction pathways associated with several growth factors, cytokines and neurokines (7). Todate, seven mammalian genes that code for different STATs have been identified, all of which encode for proteins of 750-850 amino acids long and are characterized by the presence of a DNA-binding domain followed by putative SH3 and SH2 domains. These proteins, which are normally localized in the cytoplasm, are activated when phosphorylated on a single tyrosine located around residue 700, which facilitates their dimerization and translocation to the nucleus (8). The phosphorylation of STATs is known to occur immediately after the binding of growth factors or interferons to their receptors. Since this receptor-ligand interaction was also found to result in the activation of JAK kinases, which often exist in association with cytokine receptors, it was originally thought that STATs might be substrates for JAK kinases. However, our recent studies show that interaction of IL-3 with its

receptor leads to the activation of c-Src kinase activity, which in turn facilitates the binding of c-Src to STAT-3 (9). This association leads to the phosphorylation of STAT-3, allowing this transcription factor to translocate to the nucleus. Expression of a dominant negative mutant of *src* (AMSrc) in these cells results in a block to IL-3 mediated phosphorylation of STAT-3, and its ability to bind to DNA. On the other hand, expression of a dominant negative mutant of JAK2 (JAK2KE) had no effect on IL-3-mediated activation of STAT-3. Our results also show that AMSrc does not affect the phosphorylation of JAK2, suggesting that two independent pathways mediate JAK and STAT phosphorylation events. Inhibition of c-Src activation by AMSrc, which leads to a block to STAT-3 activation, results in a dramatic inhibition of cell proliferation mediated by IL-3. These results suggest that Src family kinases mediate the phosphorylation of STATs and play a critical role in signal transduction pathways associated with myeloid cell proliferation.

It is now well established that the estrogen receptor status of primary breast tumors is of considerable value to the clinician in designing protocols for treatment. The retention of the estrogen receptor in breast tumor cells suggests a more differentiated state of the neoplasia and often suggests better prognosis and longer survival (10). An important question that remains to be addressed is the identification of the signal transduction pathway that is associated with the proliferative and metastatic potential of the ER^- breast tumors. A comparative analysis of signal transduction pathways in ER+ and ER- breast tumors in our lab led to the observation that all of the ER- breast tumors express an activated (Tyrosine phosphorylated) form of STAT-3. Interestingly, ER+ breast tumors do not express such an activated form suggesting that these tumors are not dependent on this pathway for their proliferative potential.

In our application, we proposed to examine the molecular basis for the activation of STAT-3 and design approaches to block the activation of this transcription factor and examine the effects on deactivation of this pathway on ER- breast tumor cell growth. We proposed to specifically address the following questions. (1) What is the mechanism of activation of STAT-3 in ER- breast carcinomas? (2) How is c-Src activated in ER- breast carcinomas? (3) Is there a correlation between Erb-B2 expression and STAT-3 activation? (4) Does a dominant negative mutant of STAT-3 block ER- breast tumor cell proliferation? and (5) What is the nature of genes that are activated as a result of STAT-3 activation in ER- breast carcinomas?

STAT-3 and ER-negative breast cancers:

Recent studies suggest that STATs play an important role in the proliferation of breast epithelial compartment, suggesting that this pathway may play a critical role in tumorigenesis (11). To examine the role of JAKs and STATs in the proliferation and oncogenic behavior of human breast tumor cells, we examined the activated status of STAT-3 and STAT-5 in several of the human breast carcinomas. In addition to the tumor cell lines, we used HS578BST cell line, which is derived form normal breast tissue as a control. For this, we performed a western blot analysis of the total proteins extracted from these

breast carcinomas, which have been subjected to electrophoretic separation on acrylamide gels followed by a transfer to nytran membranes **(Fig. 8).** This included a panel of ER+ and ER- tumor lines and to assess their ER status, the western blots were probed with an antibody to ER. Results from this experiment showed that MCF-7, BT474, T47D, 415, MDA-MB-330, and ZR75-30 express ER while BT20, 435, MDA-MB-435S, MDA-MB-468, MCF10F, and HTB126 were found to be ER-negative. Probing of these western blots with an antibody that recognizes the STAT-3 protein showed that all of them express approximately equal amounts of STAT-3. However, when the same blot was stripped and re-probed with an antibody that specifically recognized the Tyrosine-phosphorylated form of STAT-3 (which is activated), only the ER-negative tumor cell lines exhibited a constitutively phosphorylated form of STAT-3. These results suggest that the proliferative potential of the ER^- breast carcinomas could be mediated by the activated STAT-3 pathway.

A similar analysis of these tumor cell lines for the presence of STAT-5 revealed that this transcription factor, like STAT-3, is abundantly expressed in all of the breast carcinomas tested **(Fig. 8).** However, an analysis of its activated status showed that, unlike STAT-3, this transcription factor also exists in a constitutively phosphorylated state in both ER+ and ER- cell lines. A consistent difference seen with these two classes of tumors was a higher level of STAT-5 phosphorylation in ER+ cell lines, which was approximately 2-3 fold higher than that seen with ER-ve cell lines. In contrast to the breast tumor cell lines, HS578BST cell line did not show the presence of either STAT-3 or STAT-5 in a phosphorylated state.

Role of JAK kinases in human breast carcinomas:

To determine whether the observed phosphorylation of STAT-3 and STAT-5 in human breast carcinomas correlates with the phosphorylation status of any of the JAK kinases, we immunoprecipitated cell extracts from both ER+ and ER- cell lines with JAK-specific antibodies. These immunoprecipitates were subjected to SDS-PAGE and the western blots were probed with respective JAK antibodies and monoclonal antibody 4G10, which specifically recognizes the phosphorylated tyrosine moiety. As a positive control, we used total cell extract prepared from 32Dcl3 cells that have been stimulated with IL-3. Results from this experiment show that all of the cell lines express readily detectable levels of both JAK-1 and JAK-2 **(Fig. 9).** However, neither of these kinases were found to exist in a constitutively phosphorylated state in any of the breast carcinomas tested **(Fig. 9).** However, we could readily detect the phosphorylated form of JAK-1 and JAK-2 in our positive control, cell extracts derived from 32Dcl3 cell line that has been treated with IL-3. None of the breast tumor cell lines expressed detectable levels of Tyk-2 and JAK-3 (data not shown). These results demonstrate that the phosphorylation status of STAT-3 and STAT-5 does not correlate with the phosphorylation status of JAKs that are expressed in these cell lines.

Expression of Src kinases in human breast carcinoma cell lines

Recent studies with NIH/3T3 cells as well as 32Dcl3 cells that are transformed by

v-src have demonstrated that these transformed cells express constitutively activated form of STAT-3 (11-13). In addition, the v-Src-mediated transformation of these cells can be reversed by the expression of a dominant negative mutant of STAT-3. These studies also provided evidence that the constitutive activation of STAT-3 is preceded by the physical association of v-Src with STAT-3. A similar association of c-Src protein was seen by us in normal 32Dcl3 cells that are activated by IL-3. To determine whether the observed activation of STAT-3 in ER- breast carcinomas is mediated by Src kinases, we first examined the expression pattern of c-Src and c-Fyn in human breast carcinomas. We chose these two kinases because these two tyrosine kinases that have been previously shown by us and others to bind to and phosphorylate STAT-3. Our results from these studies demonstrate that while c-src is expressed ubiquitously in all of the breast tumor cell lines. c-fyn is preferentially expressed in ER- cell lines **(Fig. 10).**

We next examined whether c-Src and c-Fyn seen in human breast carcinoma cell lines associate with STAT-3 and whether this association is dependent on the ER status of the cell lines. For this, cell lysates were prepared form different human breast carcinoma cell lines and examined for the presence of c-Src, c-Fyn and STAT-3 by western blot analysis. We next immunoprecipitated these cell lysates with monoclonal antibodies against c-Src and c-Fyn and resolved the immunoprecipitates on 10% SDS-polyacrylamide gels. The gels were then blotted onto a nytran membrane and subjected to western blot analysis using antibodies against STAT-3. Results of these experiments show that c-Src immunprecipitates derived from ER- and ER+ breast carcinomas contain associated STAT-3 protein, even though the ER+ cells do not contain phosphorylated form of STAT-3. Similar experiments with c-Fyn show that all ER- breast carcinomas, which express this protein exhibit co-immunoprecipitation of STAT-3. These results suggest that association of Src or Fyn with STAT-3 alone is not adequate for the phosphorylation of STAT-3 and a second molecular event that has gone awry in ER- cells contributes to the phosphorylated state of STAT-3 in these cells.

Effect of dominant negative mutants of Src and Fyn on the activation of STAT-3 in ER- breast carcinomas

To definitively demonstrate that c-Src or c-Fyn mediate phosphorylation of STAT-3, we stably transfected BT-20 and 126 cell lines cells with a tetracycline-inducible dominant negative mutant of Src (AMSrc). The ATP-binding site of this c-*src* mutant was inactivated by mutation of lysine 295 to arginine rendering this protein kinase-inactive. In addition a phenylalanine substitution for tyrosine 527 prevents the intramolecular interaction between phosphorylated Y527 and the SH2 domain of this protein allowing the protein to exist in an open configuration, thus making the SH2 and SH3 domains accessible to cellular binding proteins. This protein was tagged with a peptide sequence derived from the hemaglutinin gene (the FLAG epitope) at the C-terminal end, which allowed the detection of AMSrc independent of endogenous c-Src. Following electroporation of the expression vector into BT-20 and HTB126 cell lines, the cells were selected with G418 and maintained in a medium containing 2μg/ml of tetracycline. To verify the inducible expression of AMSrc in transfected cells, they

were incubated in a medium lacking tetracycline for 48 hrs and the cell lysates subjected to western blotting using anti-FLAG antibodies. These western blotting studies showed that the expression of AMSrc protein was very leaky in these cell lines and the presence or absence of tetracycline in the medium did not affect the levels of AM-Src expressed in these cells. To determine the phosphorylation status of STAT-3 in cells expressing AMSrc, total cell lysates from normal BT-20 and HTB126 cells and AMSrc-transfected BT-20 and HTB126 cells were prepared. The lysates were immunoprecipitated with anti-STAT-3 antibodies and the immunoprecipitates subjected to western blotting. The western blots were first probed with anti-STAT-3 antibodies, which showed that all cell lines expressed equivalent amounts of STAT-3. The western blot was stripped and re-probed with anti-phospho-STAT-3 antibodies which specifically recognizes the phosphorylated form of STAT-3. These studies revealed that this antibody readily recognizes phosphorylated STAT-3 present in untransfected BT-20 and HTB126 cells while it failed to recognize STAT-3 in cell lysates derived from AmSrc-transfected BT-20 and HTB126 cells (**Fig. 11**). These results show that phosphorylation of STAT-3 in ER- breast carcinomas is blocked by AMSrc indicating that constitutive phosphorylation of STAT-3 in ER- breast carcinomas is mediated by a Src kinase.

To determine the role of dominant negative form of Fyn, we first generated a dominant negative form of Fyn by mutating the ATP-binding domain of the gene, such that an inactive kinase is produced. Transfection of this mutant into ER-negative breast carcinomas was found to result in the abrogation of STAT-3 phosphorylation, a result similar to that seen with AM-Src tansfected cells. These results suggest that either Src or Fyn can function in the activation of STAT-3 and blocking their activity results in a loss of STAT-3 phosphorylation.

Effect of dominant negative Src on proliferation and anchorage-independent growth of ER- breast carcinomas.

To investigate the effect of AMSrc on ER- breast carcinomas, we analyzed the growth pattern of BT-20/AMSrc and 126/AMSRC cells on Soft agar and compared it with untransfected BT-20 and HTB126 cell growth. AMSrc-transfected and untransfected cells were maintained in DMEM, supplemented with 10% FBS. The cells were plated at a density of $1x10^5$ cells/ml and cell viability and transformation observed for three weeks. The results of this experiment suggest that AMSrc expression in these cells leads to a loss of anchorage-independent growth and this could be attributed to the absence of an activated form of STAT-3 **(Fig. 12)**..

Effect of a dominant negative STAT-3 on anchorage-independent growth of ER- breast carcinomas.

To study the effects of a dominant negative mutant of STAT-3, we first generated an expression vector that efficiently expresses the ß-form of STAT-3 that acts as a dominant negative mutant of full length STAT-3. This gene was found to inhibit STAT-3-mediated transcriptional transactivation of appropriate reporter genes. Preliminary studies using this expression vector suggest that over-expression of

STAT-3ß results in growth inhibition and loss of anchorage-independent growth of ER-negative breast carcinomas, strongly implicating this gene in tumor cell growth.

Key Research Accomplishments:

- c-myb and A-myb genes are expressed in normal breast epithelium and regulate its proliferation.
- Over-expression of c-myb and A-myb in HC-11 mammary epithelial cells leads to the transformation of the cells.
- STAT-3 protein exists in a constitutively activated state in ER-negative breast carcinomas.
- STAT-3 phosphorylation in ER-negative breast carcinomas is mediated by Src-family of tyrosine kinases.
- ER+ breast carcinomas do not express constitutively activated form of STAT-3.
- ER+ breast carcinomas express higher levels of activated STAT-5 compared to ER-negative breast carcinomas.
- A dominant negative mutant of c-Src blocks the phosphorylation of STAT-3 in ER-negative breast carcinomas.
- A dominant-negative mutant of c-Src blocks the anchorage-independent growth of ER-negativ e breast carcinomas.
- A dominant-negative mutant of STAT-3 blocks the transactivating potential of Wt-STAT-3 and anchorage-independent growth of ER-negativ e breast carcinomas.

Reportable Outcomes:

The data obtained from the DOD grant was used in an application for NIH RO1 funding and a grant entitled "Role of Myb gene family in breast cancer" was approved for funding by NIH.

Publications:

1. A. Kumar, S.C. Cosenza, K.E. Latham, J. Litvin, D.M. Bautista and E.P. Reddy. (2001) Role of A-myb and c-myb in mouse mammary epithelial cell proliferation, cell cycle progression and transformation. Mol. Cell. Biol. (Accepted pending revision)

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3. E. P. Reddy (1998) JAKs, STATs and Src kinases: Obligate partners in cytokine signaling. Proceedings of the 9th Annual meeting of the American Association for Cancer Research. P. 33. (Meet the Expert Session).

Conclusions:

(1) Our results demonstrate that c-myb and A-myb genes play a critical role in normal growth and differentiation of estrogen and progesterone-induced proliferation of normal breast epithelial cells and over-expression of these genes leads to a pre-disposition of these cells to transformation.

(2) Our results suggest that several of the human breast carcinomas, which are ER-negative express constitutively activated form of STAT-3. STAT-3, in these tumors appears to be associated with an activated form of Src kinases, which in turn mediate the phosphorylation of STAT-3. The constitutively activated status of STAT-3, therefore serves as a good diagnostic marker for the detection of breast carcinoma development. Since STAT-3 activation appears to play a critical

role in the proliferative function of human breast carcinomas which are ER-negative, Src-STAT-3 pathway offers an excellent target for the development of new cancer therapeutic agents.

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Personnel:

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- Yen Lieu;
- Quang Nguyen;
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- M. Mallireddigari.

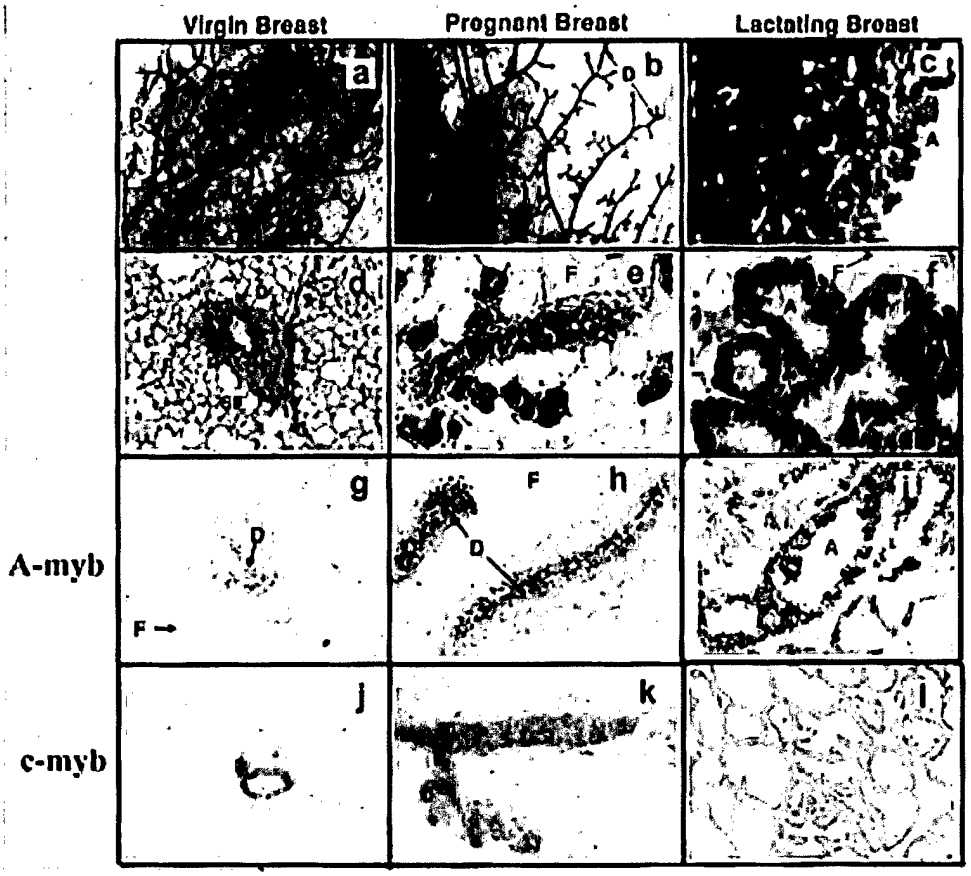


Fig. 1. In situ hybridization analysis of A-myb and c-myb expression in mouse breast tissues. Panels A-C show whole mount preparations derived from mammary glands of (a) a nulliparous wild-type mouse; (b) 10 day pregnant mouse; (c) a lactating mouse 2 days after delivery. Panels d,e and f show sections of the same tissue (200X) stained with hematoxylin and eosin. Panels Gg,h and i show hybridization of adjacent sections with a probe specific for A-myb. Panels j,k and l show similar sections hybridized with c-myb specific probe. F, Adipocytes; D, ductal epithelial cells; SF, fibroblasts; A, alveoli.

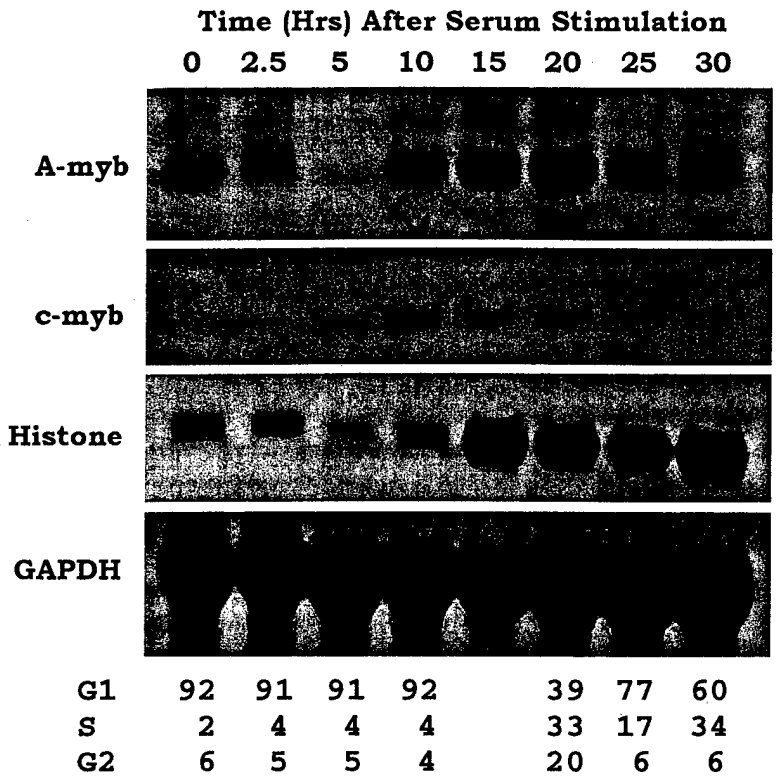


FIG. 2. Cell cycle stage-dependent expression of A-*myb* and c-*myb* mRNAs. RNA was extracted form quiescent HC11 cells at specified times after serum stimulation and analyzed by Northern blotting for the expression of A-*myb*, c-*myb*, histone H4, and GAPDH mRNAs. Parallel cultures were fixed and analyzed for DNA content by flow cytometry. The proportions of cells within each cell cycle compartment are indicated at bottom.

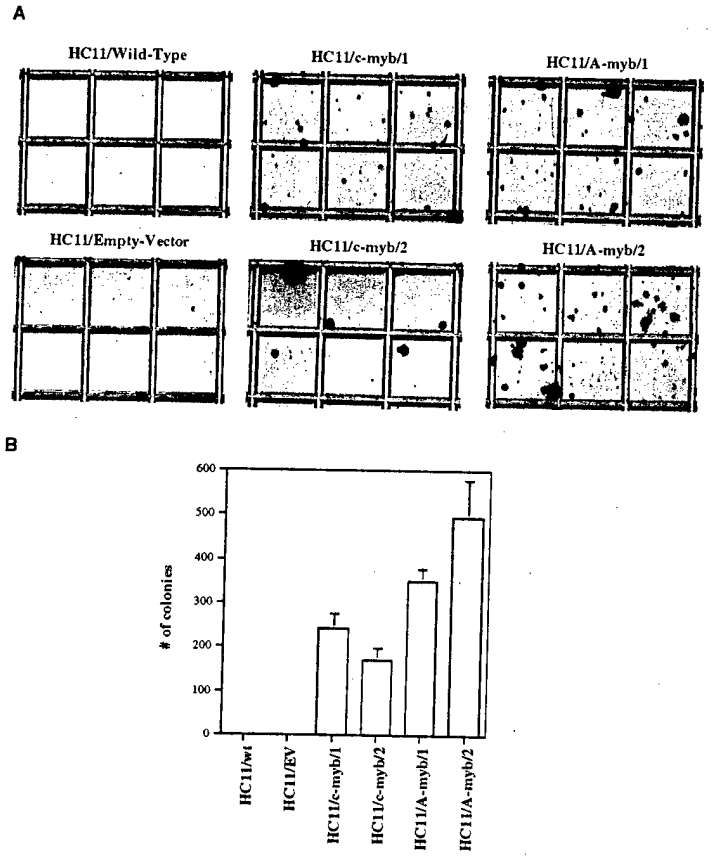


FIG. 3. Anchorage independence of HC11 cells transfected with A-*myb* and c-*myb*. (A) Anchorage-independent growth by untransfected HC11 (HC11/Wild-Type), mock-transfected (HC11/Empty-Vector), A-*myb* transfected (HC11/A-*myb*/1 and HC11/A-*myb*/2) and c-*myb* transfected (HC11/c-*myb*/1 and HC11/c-*myb*/2) cells was assayed in soft agar. Representative morphology was photomicrographed after 3 weeks of growth in complete growth medium (GM). (B) Number of colonies were measured in the 35 mm soft-agar dishes containing cells after 3 weeks of growth in growth medium. The experiment was done in triplicates.

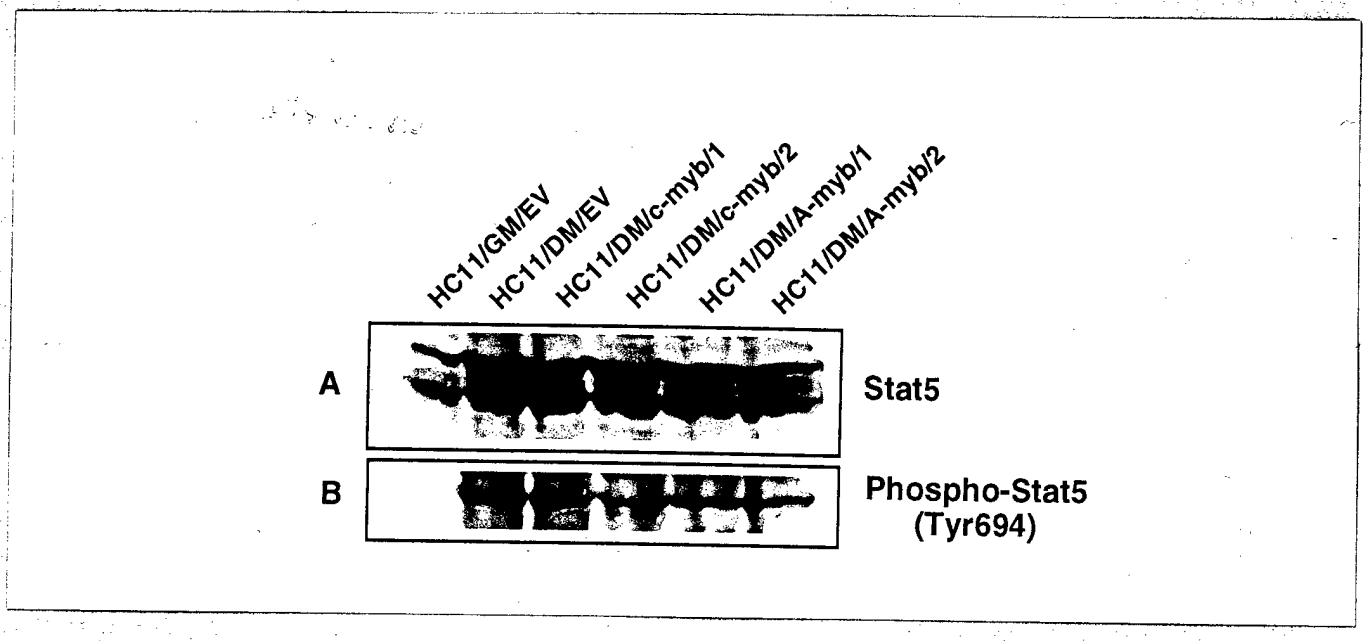


FIG. 4. Expression and activation of Stat5 in HC11 cells with deregulated expression of A-*myb* and c-*myb* during proliferation and differentiation. HC11 were induce to differentiate as follows: cells were grown to confluence and maintained in complete growth medium (GM), after which epidermal growth factor (EGF) was withdrawn and cells switched to priming medium containing hydrocortisone (HC) for 1 day and then to induction medium containing HC + prolactin (PRL) for 3 days. (A) Western blot analysis of Stat5 protein in mock-transfected HC11 cells in GM (HC11/GM/EV) and DM (HC11/DM/EV), c-*myb* transfected cells (HC11/DM/c-*myb*/1 and HC11/DM/c-*myb*/2) and A-*myb* transfected cells (HC11/DM/A-*myb*/1 and HC11/DM/A-*myb*/2). The primary antibody (which binds to both Stat5a and Stat5b) and the procedure are described in materials and methods. (B) Activation of Stat5 by tyrosine phosphorylation in different HC11 cells described in panel a. The blot was stripped and reprobed with primary antibody specific to Stat5 phosphorylated at Tyr 694.

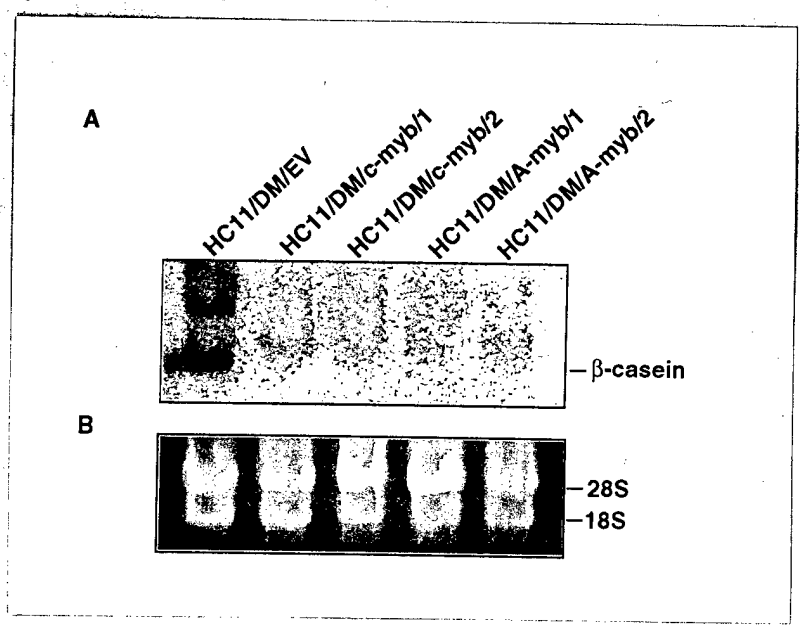


FIG. 5. Effect of ectopic expression of A-*myb* and c-*myb* on lactogenic hormone dependent differentiation of mouse mammary epithelial HC11 cells. ß-casein expression was measured in A-*myb* (HC11/DM/A-*myb*/1 and HC11/DM/A-*myb*/2) and c-*myb* (HC11/DM/c-*myb*/1 and HC11/DM/c-*myb*/2) single cell clones by Northern blot analysis of total RNA (20 µg each lane). The cells were grown to confluence and cultured for 3 days in complete growth medium with epidermal growth factor (EGF), switched to priming medium containing hydrocortisone (HC) for 1 day and then to induction medium containing HC + prolactin (PRL) for 3 days. Total RNA from empty-vector transfected cells (HC11/DM/EV) in differentiation medium is also included for comparison. As an internal control for RNA loading, gel were stained with ethidium bromide to compare levels of 18S/28S ribosomal RNA across lanes.

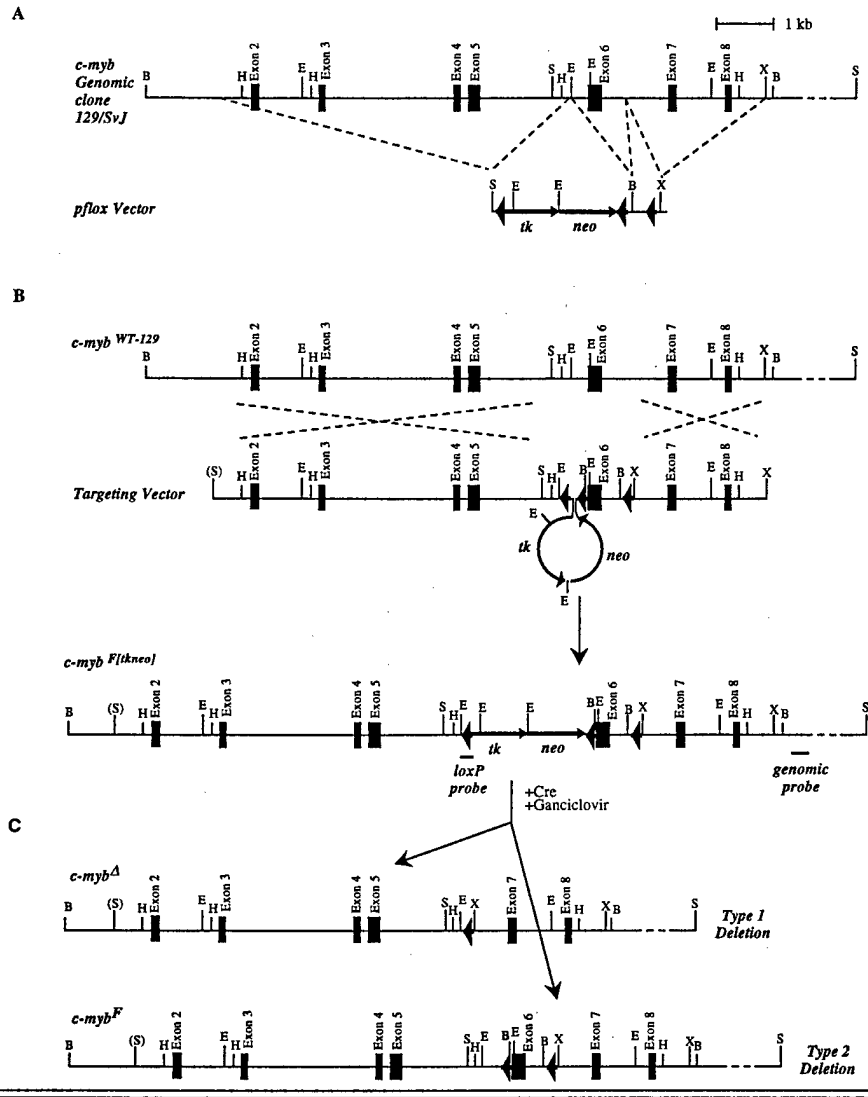


Fig 6. Mouse c-myb genomic structure and targeting vector production. (A) Restriction map of the mouse genomic clone is shown. The pflox vector is used as depicted to generate a c-myb targeting vector and contains two selectable markers tk and neo (black arrows). Thirty-four bp loxP sites are depicted as black arrowheads. (B) Homologous recombinantion between the wild-type (WT) c-myb allele in embryonic stem (ES) cells will generate the c-mybF[tkneo] allele to be used as a substrate for subsequent recombination by Cre recombinase. Positions of the probe used to determine homologous recombination and for additional genomic Southern blotting studies of targeted c-myb allele structure are depicted. (C) Production of ES cells bearing Type I and Type II deletions in the c-mybF[tkneo] allele will be generated following Cre expression and ganciclovir selection. Restriction enzyme sites: B, BamHI; E, EcoRI; H, HindIII; S, SalI; X, XhoI.Deletion of exon 6 results in the disruption of c-Myb DNA-binding domain,which renders the protein inactive.

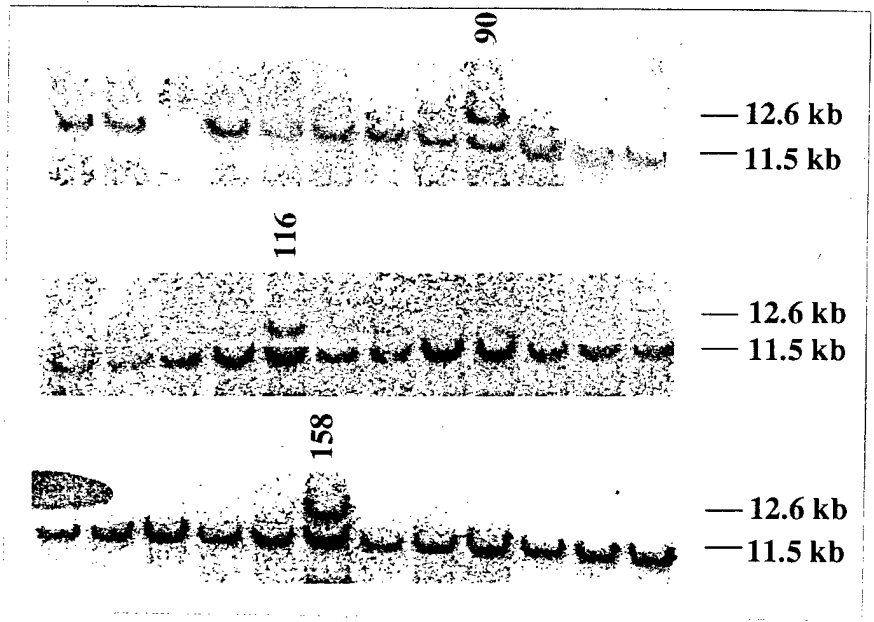


Fig.7. Homologous recombination at the c-myb locus in embryonic stem (ES) cells. Southern blot analysis was performed on ES cell DNA digested with StuI and hybridized with c-myb specific probe downstream of exon 9. Out of a total of 384 ES cell clones (G418 resistant) analyzed, 7 clones revealed the presence of conditionally targeted c-myb allele. Homologous recombinants revealed two bands: 11.5 kb (wt) and 12.6 kb (recombinant). The results shown for clones numbered 90, 116 and 158 are representative.

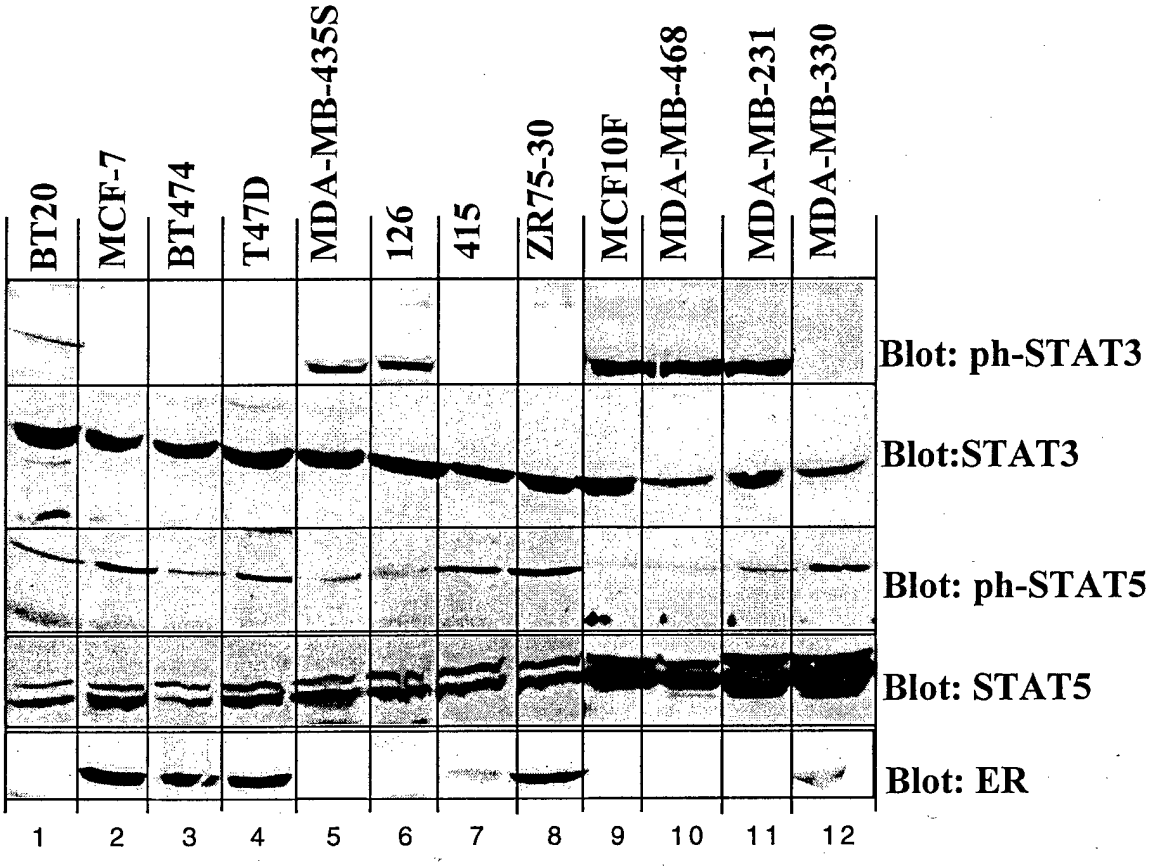


Fig. 8. Expression of STAT3, Phospho-STAT3, STAT5, Phospho-STAT5 and Estrogen Receptor (ER) in Human Breast Carcinoma Cell lines. Note the positive correlation between Phosph-STAT3 expression and lack of Er in Breast carcinomas.

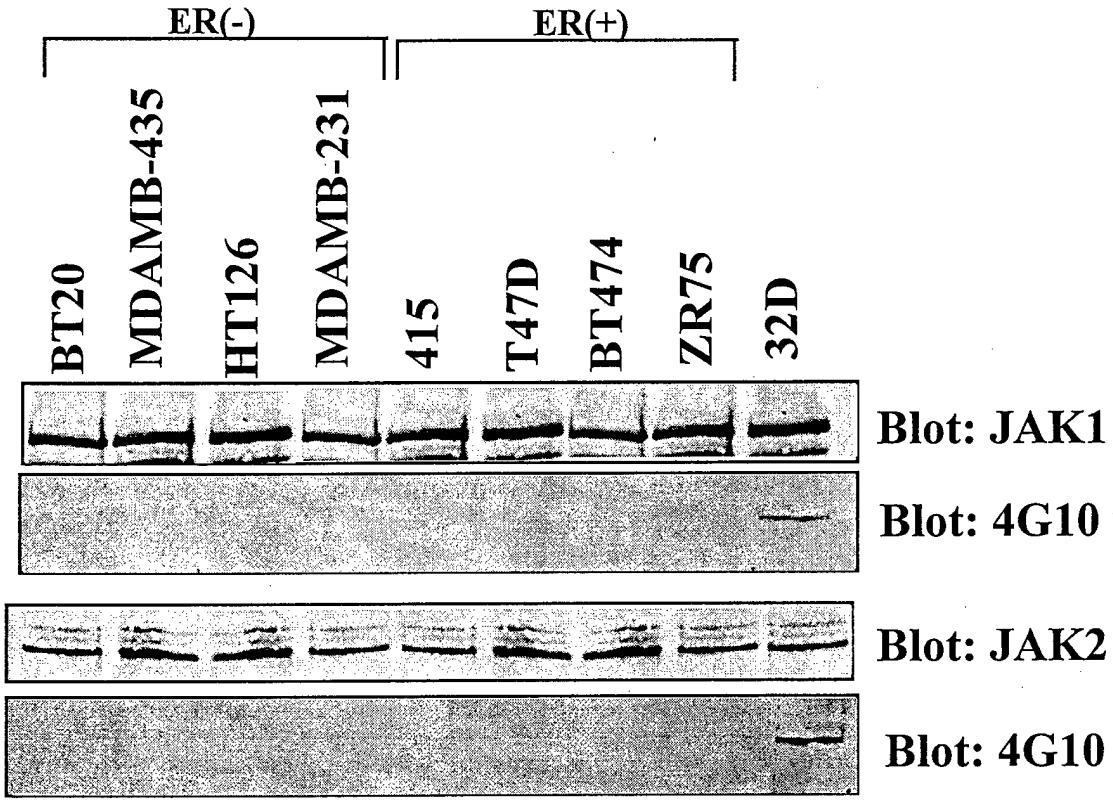


Fig. 9. Analysis of phosphorylation status of JAK-1 and JAK-2 in Human Breast carcinoma cell lines. 32D cells were used as a positive control.

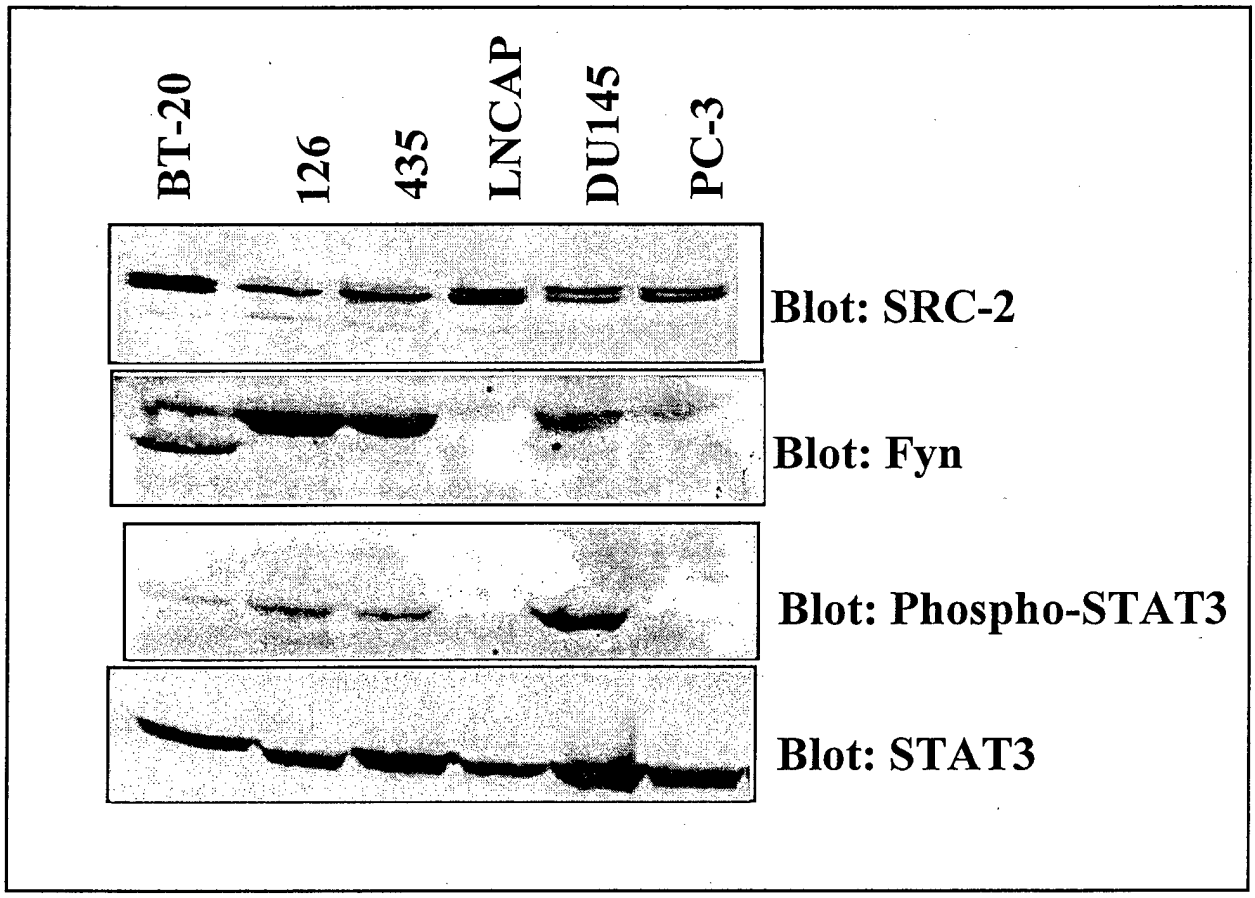


Fig. 10. Expression of Src and Fyn kinases in Human Breast carcinomas

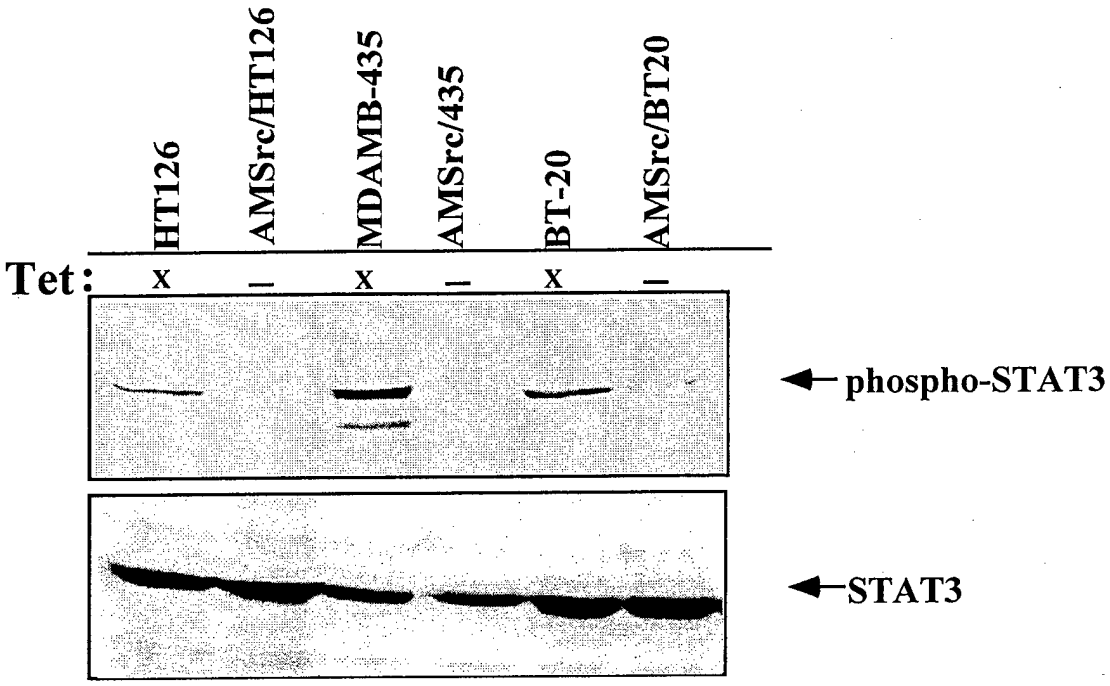
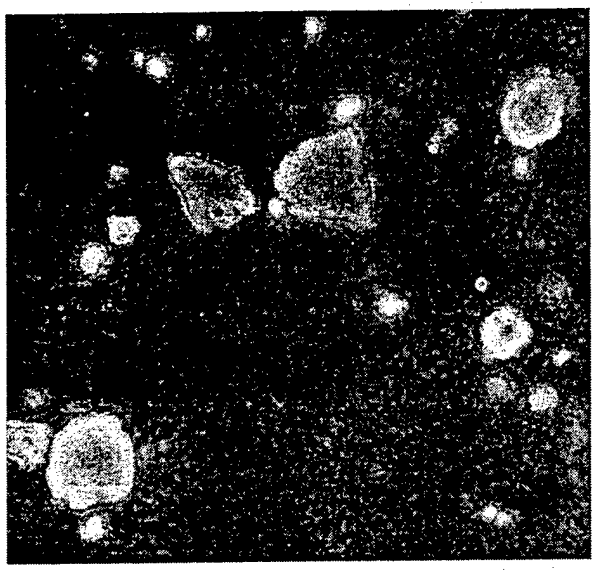
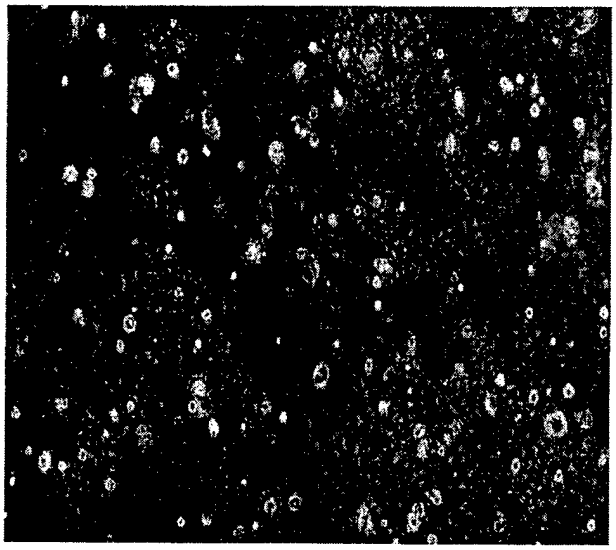


Figure 11. A dominant negative mutant of Src blocks activation of STAT-3 in ER- breast carcinomas. For direct western analysis phosphorylayed STAT3 protein in the AMSrc transfected breast cancer cell lines, total cell lysates were preapred from both normal untransfected as well as AMSrc transfected HT126, MDAMB-435 and BT20 cells. The transfected cells were washed and incubated for 48 hrs in the abscence of tetracycline before harvesting. 80 μg of total cell lysates from the indicated cells were prepared and resolved on 10% SDS-polyacrylamide gel and immunoblotted with anti-phospho-STAT3 antibody. The blot was then stripped and repbobed with anti-STAT3 antibody.



435 (Wild Type)



435 (Transfected with AM-Src)

FIG. 12. Wild-type MDAMB-435 breast carcinoma cells (ER negative) were transfected with an expression vector encoding AM-Src (a dominant negative form of Src) and the ability of control untransfected cells and AM-Src-transfected cells to grow in soft agar was assessed. While the untransfected MDAMB-435 cells grew well in soft agar and formed robust colonies (Top panel), cells transfected with AM-Src (Bottom panel) failed to grow in soft agar, suggesting that AM-Src inhibits the anchorage-independent growth of MDAMB-435 cells.